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Effects of succinylacetone on the uptake of sugars and amino acids by brush border vesicles

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Effects of succinylacetone on the uptake of sugars and amino acids by brush border vesicles. Infants with hereditary tyrosinemia excrete succinylacetone (SA) in their urine, and suffer from a reversible renal Fanconi syndrome with glycosuria and hyperaminoaciduria. Thus, we have examined the effects of 4 mM SA on rat renal brush border membrane vesicle uptake of sugars and amino acids. SA, unlike sodium maleate, significantly inhibits Na^+-dependent vesicular sugar and amino acid uptake. ^{22}Na-uptake, as well as membrane fluidity of the vesicles, are also affected by SA. Inhibition of glycine uptake by SA is reversible and competitive in nature, while α-CH_3-D-glucoside uptake is non-competitively affected. We conclude, therefore, that SA has a more complex action on the rat renal tubule than sodium maleate, and is likely a much more physiologic model for study of the human renal Fanconi syndrome.

Succinylacetone (SA) is a compound which is formed and excreted in abnormal quantities in the urine of humans affected by the autosomal recessive genetic disorder, hereditary tyrosinemia [1]. Such individuals also suffer from a secondary and reversible renal Fanconi syndrome which results in glucosuria and a generalized aminoaciduria [2, 3]. Accordingly, we have undertaken an investigation into the use of SA in creation of an animal model for the study of the biochemical basis of the Fanconi syndrome. Such a model would have distinct advantages over the maleic acid-treated animal, in which the inciting substance is not endogenous and may, therefore, bear little or no relevance to the human disorder.

We have previously reported that injection of SA into healthy, adult rats produces a constellation of urinary findings closely paralleling those of the human renal Fanconi syndrome [4]. We have also demonstrated in vitro effects of SA on sugar [4] and amino acid [5] uptake by isolated rat renal tubule fragments. Our earlier data indicate reversible effects of SA on oxygen consumption by intact tubule and isolated mitochondria, and inhibition of α-methyl-D-glucoside and α-amino-isobutyric acid uptake by isolated renal tubules.

In order to further characterize the nature of the transport inhibition caused by SA in the renal tubule, we have extended our studies to an examination of the effects of SA on sugar and amino acid uptake by rat renal brush border membrane vesicles. This preparation permits direct examination of transport events at the level of the renal proximal tubule cell membrane in isolation, thus eliminating concerns regarding substrate metabolism, oxygen consumption, etc. The results of these studies comprise the basis for the present report.

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Methods

Adult male Sprague-Dawley rats weighing 150 to 175 grams were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts, USA). Animals were provided water and Purina chow ad libitum. D-[1-^{14}C]-glucose (54.6 mCi/mmol), methyl-α-D[U-^{14}C] glucose (250 mCi/mmol), glycine-[2-^{14}C] (49.2 mCi/mmol), L-[U-^{14}C]-lysine (354.6 mCi/mmol) and $^{22}NaCl$ (450 to 1000 mCi/mg) were purchased from New England Nuclear (Boston, Massachusetts, USA). Unlabeled substrates were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Succinylacetone was purchased from Calbiochem (San Diego, California, USA), made to 200 mM stock in water (pH 7.0) and stored at −20°C until used. All reagents were purchased from commercial sources and were of the highest grade obtainable.

Membrane preparation

Animals were killed by stunning and decapitation. The kidneys were removed rapidly, decapsulated and placed in normal saline on ice. Cortical slices (0.5 mm) were made with a Stadie-Riggs microtome and the tissue weighed. Brush border membrane vesicles were then prepared using the method of Booth and Kenny [6] modified as described by Weiss, et al [7]. The final membrane pellet was suspended in THM buffer, pH 7.4 (2 mM Tris/HEPES + 100 mM mannitol) to a protein concentration of 0.3 to 0.4 mg/ml as determined by the method of Lowry, et al [8].

Substrate uptake studies

All measurements of vesicular uptake were performed at 25°C using Millipore filtration on HAWP filters (0.45 μm) as previously described [9, 10]. The vesicles demonstrated osmotic activity and did not metabolize substrate. Results are expressed as nmoles (or pmols) of substrate uptake/mg protein. Efflux was examined by performing three-minute incubation of vesicles under 100 mM Na-gradient conditions with 0.06 mM glycine alone, or with 0.12 mM glycine + 4 mM SA. In the latter case, the higher substrate concentration was chosen, based on

the uptake curves, in order to equalize intravesicular concentration with the control. Following the three-minute incubations, the vesicles were instantaneously transferred to a second set of tubes containing THM buffer + 100 mM NaCl only, or THM buffer + 100 mM NaCl + 4 mM SA. Sampling was performed at 0, 15 and 30 seconds, and 1, 3, 10 and 20 minutes following this transfer. Filters were dried and counted and data calculated as cpm/mg. Results were expressed as percent cpm/mg remaining with time, compared to the 0 time, taken as 100%. Data were examined for statistical significance by Student's *t*-test, using an APPSTAT program and an Apple IIE computer.

Sodium uptake studies

The effects of SA on the entry of $^{22}Na^{+}$ into the membrane vesicles was examined. These studies were performed using vesicles suspended in 100 mM NaCl + THM buffer at pH 7.4 for 60 minutes at 25°C. To initiate the incubation, 480 μl of vesicle suspension (=0.2 mg protein) was added to a mixture containing 10 μl of 200 mM SA or 10 μl THM buffer, 5 μl of 6 mM glycine and 5 μl (0.5 μCi) of $^{22}NaCl$ to bring the total volume to 0.5 ml, with final concentration of 0.06 mM glycine and 100 mM NaCl with or without 4 mM SA. The entry of sodium was determined from 15 seconds to 20 minutes, after which the incubation was stopped by rapid filtration of the mixture, followed by washing the filters with 5 ml of ice-cold buffer containing 154 mM choline chloride, 100 mM mannitol and 2 mM Tris/Hepes, pH 7.4. Filters were air-dried and assayed for radioactivity in a Beckman Gamma 4000 γ-counter. Study of the effects of medium osmolarity on the entry of $^{22}NaCl$ into vesicles was performed by incubation of 100 mM NaCl-equilibrated membranes with increasing amounts of sucrose (equivalent to 150 to 400 mOsmol) in THM buffer pH 7.4 at 25° for 60 minutes and uptake studies carried out as described above.

Fluorescence polarization studies

Membrane suspensions were labeled with the lipid-soluble fluorophore, 1,6-diphenyl-1,3,5-hexatriene (DPH), according to methods previously described [11]. Briefly, 100 μg of membranes was added to 3 ml of DPH in phosphate buffered saline (PBS), (1:2000, vol/vol) and incubated at 37°C for 30 minutes. To examine the effects of succinylacetone (SA), 30 μl of SA in PBS was added to the suspension to give a final concentration of 4 mM, and incubated an additional five minutes. Measurements were performed with a Shimadzu fluorescence spectrophotometer Model RF-540 equipped with an automatic polarizer. Temperature was controlled and determined by a Perkin-Elmer model C570-0701 temperature programmer (Perkin-Elmer, Norwalk, Connecticut, USA). Excitation wavelength was 360 nM, emission wavelength was 430 nM. Readings were taken by recording emission intensities parallel and perpendicular to the plane of excitation ($I_{\parallel}$ and $I_{\perp}$).

The polarization of fluorescence was expressed as the fluorescence anisotropy, r, which is derived from the Perrin equation:

$$r = [I_{\parallel} - I_{\perp} C]/[I_{\parallel} + 2I_{\perp} C]$$

The correction factor, C, equal to $I_{\parallel}'/I_{\perp}'$, the primes indicating excitation polarized in a parallel direction, was used to correct for the contribution due to scattering. Scattering corrections were applied to all measurements.

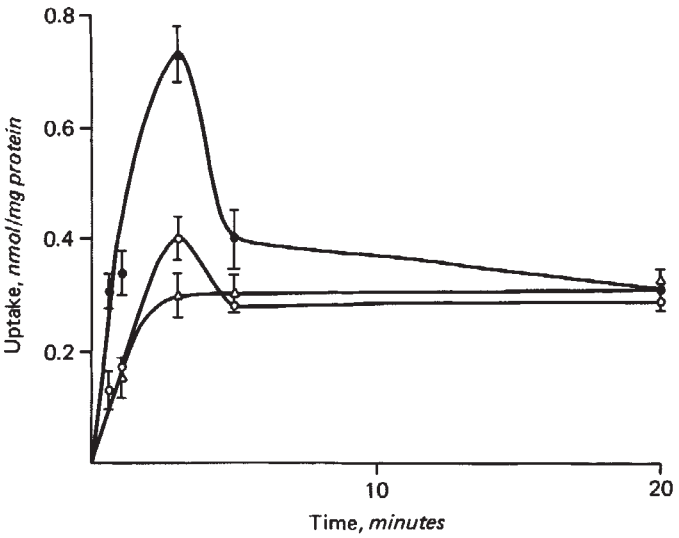

Fig. 1. *Effect of SA on glycine uptake by brush border membrane vesicles.* Vesicles were incubated in the presence of 0.06 mM glycine containing 0.1 μCi ^{14}C-glycine/tube. Control (●) and 4 mM SA-treated (○) vesicles were incubated in 100 mM NaCl gradient; 100 mM NaCl sodium-equilibrated (△) vesicles were allowed to stand at 25°C for 20 minutes prior to study. All points represent the means of 4 separate experiments with at least 12 separate determinations and are shown ±SE.

Values of r∞, the limiting hindered anisotropy, for DPH were calculated from r values as described by van der Meer, van Hoeven and van Blitterswijk [12] where:

$$r\infty = 4/3\ r - 0.1$$

For examination of the effects of temperature, preparations were cooled to 5°C and the fluorescence polarization was measured every 0.5°C as the suspension was warmed at a rate of 1°C/min to 45°C.

Results

Effect of SA on amino acid and sugar uptake

Uptake of 0.06 mM glycine by membrane vesicles incubated with an inwardly directed 100 mM NaCl gradient reached the peak overshoot at three minutes. Vesicles allowed to equilibrate in 100 mM NaCl prior to the start of incubation with glycine exhibited no overshoot. Addition of 4 mM SA to the vesicles significantly reduced the NaCl gradient-generated overshoot ($P \leq 0.0001$). The peak of the overshoot occurred at three minutes both in the absence and presence of SA (Figure 1). Even after 30 seconds of incubation the uptake by SA-treated vesicles was significantly reduced compared to the controls ($P < 0.0001$). All incubations resulted in similar equilibrium uptake of 0.06 mM glycine by 20 minutes, indicating an equivalent intravesicular volume of distribution. Glycine efflux from preloaded vesicles in the presence of 4 mM SA did not differ from controls; in both cases, the T1/2 was 3.4 minutes.

Table 1. Effect of 4.0 mM succinylacetone on membrane uptake of sugars and amino acids

Substrate	Concentration mM	Uptake nmol/mg Control	4 mM SA	P
Glycine	0.06	0.729 ± 0.045	0.398 ± 0.054	0.0002
	1.1	5.83 ± 0.361	3.8 ± 0.413	0.001
L-lysine	0.06	0.434 ± 0.021	0.24 ± 0.027	0.02
	2.1	13.2 ± 1.002	6.80 ± 0.931	0.001
αCH_3-D-glucoside	0.06	0.780 ± 0.069	0.571 ± 0.072	0.02
D-glucose	0.06	0.623 ± 0.043	0.212 ± 0.061	0.001

Vesicles were incubated for 3 minutes at 25°C in 100 mM NaCl gradient, with or without added 4 mM SA. Uptake values are given as the means of at least three experiments including 9 separate determinations ± standard error. *P* values were determined as described under **Methods**.

Table 2. Reversibility of 4 mM SA inhibition of 0.06 mM glycine uptake in the membrane vesicle

Time of incubation	Uptake nmol/mg Control	4 mM SA-treated
3 minutes	0.655 ± 0.056	0.654 ± 0.054
20 minutes	0.351 ± 0.029	0.339 ± 0.020

Membrane vesicles were preincubated with or without 4 mM SA for 30 seconds, centrifuged and resuspended in fresh THM buffer without SA. Uptake was studied as outlined under **Methods**. Values shown represent the means ± SE of 3 experiments including 9 separate determinations.

A similar phenomenon was observed with other substrates as SA caused a reduction in the amount of material taken up by three minutes compared to controls when the vesicles were incubated in the presence of an inwardly directed 100 mM NaCl gradient (Table 1). Further, with glycine and lysine, where uptake is known to occur simultaneously via at least two different transport systems, succinylacetone exerted an inhibitory effect on uptake at both low and high substrate concentrations.

In view of the fact that transport inhibition caused by 4 mM SA in the isolated tubule is reversible, we were concerned that this was due to physical damage to the membrane. Thus, we further examined the basis for our observations by testing for the reversibility of SA mediated transport inhibition in isolated membrane vesicles. Vesicles were pre-incubated in either THM or THM + 4 mM SA for 30 seconds at 25°C. This time interval was chosen as the earliest time associated with demonstrable uptake inhibition. The vesicles were then recentrifuged, the supernatant discarded and the vesicles in each tube resuspended in fresh THM with no SA, and the uptake of 0.06 mM glycine examined. The results, shown in Table 2, clearly demonstrate no differences in glycine uptake between control and SA-exposed vesicles.

Effect of 4 mM SA on concentration-dependent uptake of glycine

In order to better assess the nature of the inhibition by SA on glycine uptake, we examined changes occurring in concentration-dependent uptake by membrane vesicles. The membranes were incubated for 30 seconds at 25°C in 100 mM NaCl with or without 4 mM SA. Glycine concentration was varied from 0.021

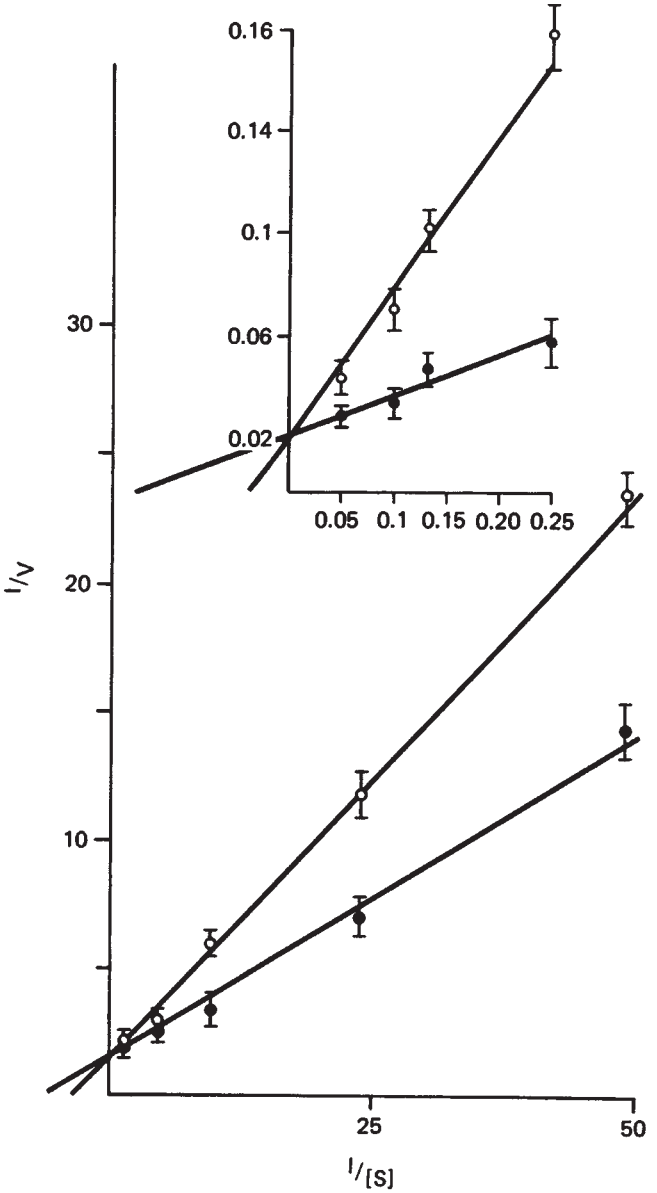

Fig. 2. *Effect of SA on concentration-dependent uptake of glycine by brush border membrane vesicles.* Vesicles were incubated in 100 mM NaCl gradient with (○) and without (●) 4 mM SA for 30 seconds at 25°C over a concentration range of 0.021 to 0.21 mM glycine and 0.70 to 20 mM glycine (inset). Velocity (V) is expressed as nmol glycine taken up per mg protein/30 sec; substrate concentration (S) is expressed in mmol/liter. Points represent the mean values of 5 separate experiments with at least 15 separate determinations.

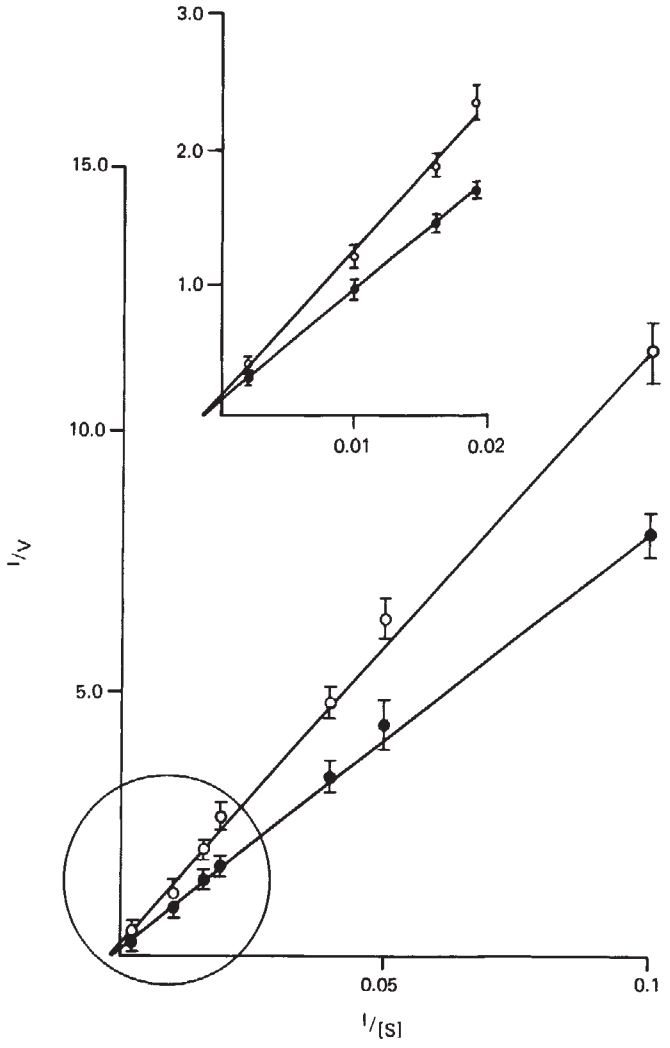

Fig. 3. *Effect of SA on concentration-dependent uptake of α-CH_3-D-glucoside by brush border membrane vesicles.* Vesicles were incubated in 100 mM NaCl gradient with (○) and without (●) 4 mM SA for 30 seconds at 25°C over a concentration range of 10 μM to 0.51 mM sugar. V is expressed as nmol hexose taken up per mg protein/30 sec; S is in μmol/liter. Points are the mean values of 4 separate experiments with at least 12 separate determinations. Data shown in inset represent a magnified view of those points circled and are the mean values of the above.

mM to 20.0 mM in order to examine the separate uptake systems known to exist for this amino acid. Concentration-dependent uptake over a substrate range from 0.021 to 0.21 mM appeared to be competitively-inhibited by 4 mM SA (Fig. 2), the apparent transport K_M values calculated as 0.18 (±0.013) mM, and 0.33 (±0.020) mM in the absence and presence of SA, respectively ($P < 0.05$). Inhibition at higher substrate concentrations was also competitive in nature (Fig. 2 inset), the K_M values calculated as 6.78 (+0.93) mM for control and 28.57 (±1.67) mM SA-treated vesicles ($P < 0.01$).

Effect of 4 mM SA on concentration-dependent uptake of α-CH^3-D-glucoside

Concentration-dependence of α-methyl-D-glucoside uptake was assessed with and without addition of 4 mM SA. Vesicles were incubated, as for the glycine studies above, for 30 seconds over a substrate concentration range of 10 μM to 0.51 mM in 100 mM NaCl at 25°C. Data were plotted by the double reciprocal method, and are shown in Figure 3. Thus, in this substrate concentration range, SA exerts a non-competitive inhibitory effect on αMG uptake. The apparent transport K_M value for both control and SA treated vesicles was 1.0 (±0.081) μM, while the V_{max} decreased from 8.25 (±0.631) nmol/mg/30 sec in controls to 6.25 (±0.513) nmol/mg/30 sec in the SA treated membranes ($P < 0.01$).

$^{22}Na^+$ influx studies

As SA exerts an inhibitory effect on Na^+-stimulated substrate transport, it was necessary to determine the influence of SA on transmembrane Na^+ movement. Pre-equilibration of the vesicles in 100 mM NaCl was performed to minimize non-specific Na^+-binding to the membranes [13]. Osmotic studies demonstrated ^{22}Na-binding to the membranes of less than 12% of the total. Thus, the observations represent the entry of tracer into the vesicular space with time. Control samples demonstrated a rapid rate of $^{22}Na^+$-entry, achieving 35% of the 20-minute total within 60 seconds of incubation. In contrast, addition of 4 mM SA caused a decrease in this rate of entry, reducing the 60 second value to less than 10% of the 20-minute total. This difference was highly significant ($P < 0.0001$); 20-minute totals were 3.45 ± 0.01 pg ^{22}Na/mg protein and 3.47 ± 0.12 pg ^{22}Na/mg protein for control and SA-treated vesicle, respectively. The curves representing ^{22}Na-entry over 20 minutes are shown in the presence and absence of 4 mM SA in Figure 4.

Fluorescence polarization studies

Figure 5 shows fluorescence anisotropy, r, as a function of temperature; higher values of r indicate a lower membrane fluidity. Incubation of membranes with 4 mM succinylacetone results in an increased membrane fluidity over the entire temperature range examined. There do not appear to be any "break points" in these lines, indicating that throughout this temperature range, membranes alone or with SA do not undergo phase transition, as determined by fluorescence polarization of DPH.

The data in Table 3 show mean fluorescence polarization of DPH, and r∞ which is a static component of r, related to the lipid structure of the membrane. These values are thought to more closely provide information on r for lipid soluble probes such as DPH, and may be the best indicator of the relationship between membrane lipid structure and its influence on membrane transport processes. Incubation of membranes with SA results in a significant decrease in r, as well as a decrease in the derived value of r∞, indicating a fluidizing effect of SA on these membranes.

Discussion

The renal brush border membrane vesicle preparation offers distinct advantages over an intact cellular preparation for investigation of nephrotoxic agents: substrate metabolism may be neglected in interpretation of data, and the study of membrane transport events can be confined to a particular surface of the bipolar tubular epithelial cell. Thus, we have turned to the use of this methodology in order to extend our efforts toward characterization of a recently described animal model for the

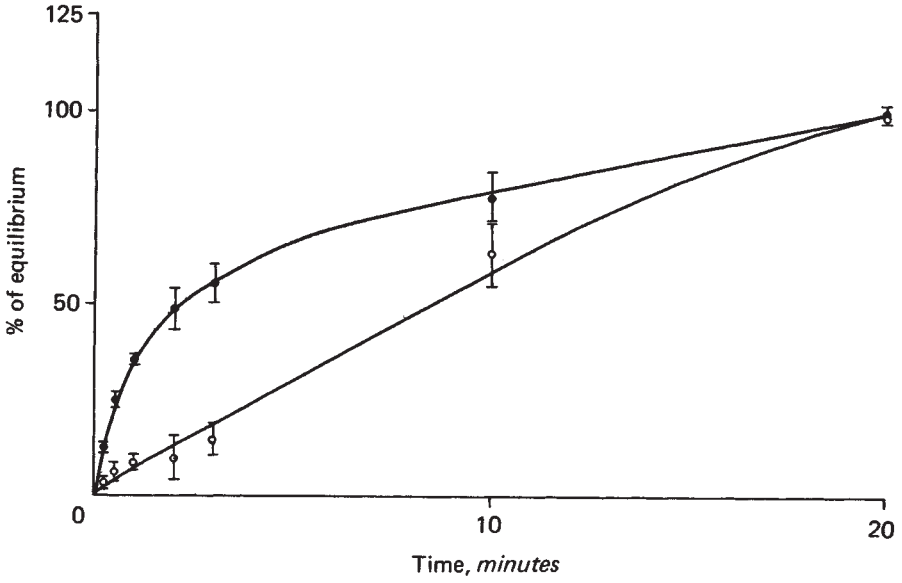

Fig. 4. *Effect of SA on ^{22}Na-entry into membrane vesicles.* Vesicles pre-equilibrated in 100 mM NaCl were incubated with 0.5 μCi $^{22}NaCl$ with (○) and without (●) 4 mM SA, as detailed in **Methods.** Points are shown as the mean percentages (±SE) of 20-minute uptake versus time in minutes. Twenty-minute uptake values did not differ significantly for control and SA-treated vesicles. Data are derived from three separate experiments with at least 15 separate determinations.

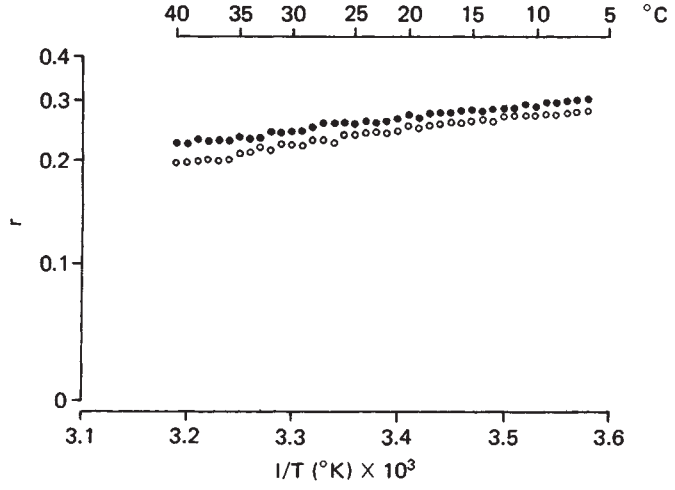

Fig. 5. *Representative Arrhenius plot of the fluorescence anisotropy, r, for DPH of renal brush border membranes incubated with (○) or without (●) 4 mM succinylacetone according to techniques in* **Methods.**

Table 3. Fluorescence polarization of DPH in rat renal brush border membranes

	Temperature	Mean fluorescence anistropy r	Limiting hindered anistropy r_∞
Membranes with DPH	15°C	0.274 ± 0.004[a]	0.265
	25°C	0.249 ± 0.003[b]	0.232
	37°C	0.222 ± 0.003[a]	0.196
Membranes with DPH + 4 mM succinylacetone	15°C	0.215 ± 0.02	0.187
	25°C	0.209 ± 0.01	0.179
	37°C	0.196 ± 0.01	0.161

Fluorescence polarization data determined according to the methods described in the Materials and Methods section. Results significantly different from values obtained at the same temperature with membranes incubated with 4 mM succinylacetone at $^{a}P < 0.05$, and $^{b}P < 0.02$. Significance was determined using the Student's t-test; values shown are mean ± SEM determined from 3 membrane vesicle populations.

human renal Fanconi syndrome based on the compound succinylacetone [4, 5]. In the intact rat, 4 mM SA causes glucosuria and aminoaciduria, observations which have been shown to be consistent with SA-induced transport inhibition of α-CH_3-D-glucoside and α-aminoisobutyric acid in the isolated rat renal proximal tubule fragment. Thus, an examination of SA-induced transport events occurring at the brush border surface was a logical sequential step, performed using renal brush border membrane vesicles.

Based on our earlier observations in the isolated tubule that SA reversibly reduces O_2 consumption while affecting membrane transport of sugars and amino acids in a non-competitive fashion [4, 5], we anticipated finding little, if any, effect of SA on uptake by the brush border vesicle. However, the present data indicate that, in the presence of 4 mM succinylacetone, the vesicle uptake of several substrates is significantly reduced. Our data also show the reversible nature of this transport inhibition. Such findings point to an important difference between the SA and maleic acid-induced models: in the latter, maleate-treated isolated tubules show transport inhibition of sugars [14] and amino acids [15] without demonstrable effects on membrane vesicle uptake [16]. Thus, the transport-related actions of SA on the intact tubule cell appear to be more complex than those of maleate.

Examination of the effects of SA on concentration-dependent uptake of α-CH_3-D-glucoside and glycine revealed evidence of inhibition in the vesicle, an observation which is entirely consistent with our earlier findings in the isolated rat renal tubule [4, 5]. Thus, the SA-induced transport inhibition demonstrated in the intact cell is likely due to the combined effects of succinylacetone on transmembrane flux, per se, and depression of O_2 consumption for energy generation as previously demonstrated [4]. Such factors could adequately explain the occurrence of glucosuria and aminoaciduria in SA-treated rats

in vivo [4, 5]. Since our studies were carried out over concentration ranges corresponding to normal blood levels in the rat, we believe that our observations may reflect actual physiologic events attributable to SA.

Since the sodium ion is known to be key in active uptake of sugars and amino acids, either for substrate binding or for the entry process itself, we examined the effect of succinylacetone on Na^+-entry into the membrane vesicle. Our observations indicate that actual entry of $^{22}Na^+$ into the vesicle is significantly retarded in the presence of 4 mM SA. The possibility that sodium binding characteristics of the membrane were affected by SA was eliminated by a 60-minute equilibration of the vesicles in 100 mM NaCl. The possibility that SA causes an accelerated collapse of the sodium gradient can be disregarded, based on our observation that the initial inward flux of tracer is slowed when SA is added to the membranes. Retardation of sodium entry, under our experimental conditions, could have occurred through SA-mediated effects on Na-H antiport, the glycine-sodium cotransport mechanism, or a combination of the two. That the observed decrease in entry rate cannot be attributed to increased outward flux is supported by glycine efflux studies, in which no effect of SA on the rate of glycine loss from preloaded vesicles could be demonstrated.

The fluorescence data are consistent with the inhibitory effect of SA on membrane transport of both sugars and amino acids. These findings suggest that the transport inhibition may be related to the physical environment of the cell membrane in which the carrier operates. Membrane lipid fluidity, as evaluated by fluorescence spectrophotometry, exerts a regulatory effect on intrinsic membrane proteins such as alkaline phosphatase, Na^+-K^+ ATPase and transport proteins, and may be partly responsible for resolution of the neonatal aminoaciduria with increasing age and diminished membrane fluidity [13, 17–19]. While it is generally accepted that membrane fluidity, largely determined by membrane cholesterol and phospholipid content and fatty acid saturation [20–22], changes with altered membrane lipid content, fluidity changes in the absence of compositional changes can result in altered transport kinetics for glucose [23] and H^+ [24].

Incubation of renal brushborder membranes with SA results in a parallel displacement of the line describing fluorescence anisotropy and temperature. This is similar to the shift seen in rat renal brushborder membrane vesicles with age, where the membranes progressively become more rigid with age [13]. These findings indicate the importance of understanding the relationship between membrane structure and physical characteristics, and a functional aspect such as solute transport.

A recent demonstration by Molitoris and Kinne [25] of the effects of renal ischemia on rat renal cortical brush border membrane structure and function provides support for our observations. These workers found that vesicles prepared from kidneys of rats made ischemic in situ evidenced a 50% decrease in the V_{max} of Na^+-dependent glucose uptake (non-competitive inhibition) with reduced binding capacity of the carrier, and a correlated increase in membrane fluidity; as in our present studies, decreased uptake was related only to influx. However, sodium-dependent uptake of alanine by these vesicles was unaffected, in contrast with our observations with glycine and other amino acids. This difference could be due to various factors, perhaps most likely differing membrane structural changes which vary in degree and nature depending upon the perturbation. This speculation is supported by functional changes persisting in vesicles prepared from ischemic rat kidneys [25], compared to the reversible effect of SA on glycine uptake by vesicles prepared from normal rat kidneys in our studies. We have not examined the possibility that chronic SA administration might induce irreversible membrane structural functional changes.

We have now demonstrated that SA, unlike maleate [16], holds inhibitory properties for rat renal proximal tubular epithelium at both the membrane and mitochondrial levels, and this inhibition affects the transport of sugars and amino acids, as well. These factors are highly significant to the study of a model system for the human renal Fanconi syndrome, inasmuch as SA is endogenously produced in humans while maleate is not. Thus, further characterization of the succinylacetone model would be of great value to an understanding of the biochemical basis for the renal tubular dysfunction seen in the human Fanconi syndrome.

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